IJBST

ISSN: 0974 – 3987
IJBST (2012), 5(10):49-53

Intranasal insulin modulates hippocampal amyloid ß (Aß) levels by regulating glycogen synthase kinase (GSK)-3α in diabetic rats

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ABSTRACT:
Diabetes and Alzheimer disease (AD), two age related diseases, are on the rise across the globe. Recent studies have demonstrated diabetes as a major risk factor for AD. Disturbances in insulin signaling pathway appear to be the connecting link between the two illnesses. In the present study, we characterized a non-transgenic streptozotocin induced type 1 diabetic rat model and the effect of treatment with insulin. Five weeks of insulin deficient diabetes significantly reduced the insulin degrading enzyme activity and increased glycogen synthase kinase (GSK)-3α levels. Treatment with insulin resulted in suppression of GSK-3α levels, one of the major modulators of Aß synthesis. These findings strengthen intranasal insulin treatment as a therapeutic modality in reducing the AD-like features in the brain.

Key words: Alzheimer disease, diabetes, glycogen synthase kinase-3, insulin, insulin degrading enzyme, streptozotocin

INTRODUCTION

Alzheimer's disease (AD), the commonest form of dementia, is a neurodegenerative disorder affecting the cortex and limbic system, leading to progressive cognitive decline and has been described as the pandemic of 21st century [1]. AD is characterized by several neuropathological markers, including senile plaques containing aggregated amyloid ß (Aß) and neurofibrillary tangles comprising of hyperphosphorylated tau in the form of paired helical filaments. While the less frequent, early onset, familial AD is caused by mutations in one or more of the genes [2] encoding the precursor of Aß i.e. amyloid precursor protein (APP) or the APP processing aspartyl protease complexes involving presenilin 1 or 2, the more prevalent, late onset, sporadic AD might be caused by environmental and/ or lifestyle factors [3]. Numerous recent studies have shown that patients with diabetes exhibit an increased risk of developing AD [4]. An association between AD and diabetes has been suggested with AD patients showing impaired insulin function [5] and diabetic patients exhibiting cognitive deficits [6]. Disturbance of the insulin signaling pathway is emerging as a common feature of both AD and diabetes. Insulin, acting at insulin receptors, activates signal transduction via the phosphatidylinositol 3-kinase – protein kinase B pathway. Downstream of this pathway lies glycogen synthase kinase-3 (GSK3) existing as two isoforms, α and ß. Their activity is modulated by phosphorylation at serine 21 and serine 9 respectively [7]. These two isoforms have been shown to play an important role in AD pathology wherein GSK-3α enhances the production of Aß peptides [8] and GSK-3ß catalyzes the hyperphosphorylation of tau protein [9]. Insulin degrading enzyme (IDE), the major protease responsible of insulin degradation, cleaves Aß in neuronal and microglial cells. Thus, the two substrates of IDE, Aß and insulin, connect AD and diabetes [10].

We have recently demonstrated that intranasal administration of insulin to streptozotocin (STZ) induced diabetic rats resulted in reduction of hippocampal Aß levels [11] which is in accordance with other recent reports [12-14]. To further investigate the mechanism of insulin mediated Aß reduction in the CNS, in the current study, we have monitored the influence of intranasally administered insulin on diabetic rats with special reference to the key modulators of Aß levels in the hippocampus viz., GSK-3α which promotes Aß synthesis and IDE, which facilitates Aß degradation.

MATERIALS AND METHODS

Materials:

Streptozotocin, insulin (human, recombinant), were purchased from Sigma Aldrich, Bangalore. ELISA kit for estimation of GSK-3α was purchased from Uscn Life Science Inc, Wuhan, P.R. China. Insulysin/IDE Immunocapture Activity assay kit was obtained from Calbiochem. The RNA isolation kit and cDNA synthesis kit were purchased from Bioline Ltd, U.K. All other reagents used were of analytical grade and obtained locally. Streptozotocin induced diabetic rats were developed and maintained as described earlier [11]. All the protocols used in this study were approved by Institutional Animal Ethics Committee.

Intranasal insulin administration and collection of the tissue:

After 4 weeks of hyperglycemic state, for six consecutive days, insulin was given intranasally (5 IU/ day/ animal). Following 24 h after the final dose, the rats were sacrificed by cervical dislocation and the

ISSN: 0974 – 3987
IJBST (2012), 5(10):49-53

brain removed. Hippocampi were dissected, snap frozen in liquid nitrogen and stored -80^{o}C until further analysis.

RT-PCR analysis of hippocampal mRNA:

Total RNA from 30 mg of the tissue was extracted using the commercial kit (Bioline, UK) as per the manufacturer's instructions. Integrity of RNA preparation was assessed by 1.5 % agarose – formaldehyde gel electrophoresis. Total RNA (2µg) was used to prepare cDNA (cDNA synthesis kit, Bioline, UK) by mixed priming strategy as per the manufacturer's instructions. The cDNA was amplified by PCR with the following gene specific primers for 18 S RNA [forward primer (fp) – 5'-AATCCGATAACGAACGAGA-3', reverse primer (rp) – 5'-ATCTAAGGGCATCACAGACC-3'], IDE [fp- 5'-CCTCAAAGACTCACTCAACG-3', rp- 5'-TAGCAAAATTGGCTGTTTGT-3'] and GSK-3α [fp-5'-TCAAGGCTCTCCCCACTAGA-3', rp- 5'-GTGAGGAGGGATGAGAATGG-3'] The size of the amplification product was 140 bp for 18 S RNA, 130 bp for IDE and 165 bp for GSK-3α. PCR products were electrophoresed on 2% agarose gel, amplification products stained with ethidium bromide and quantitated using Quantity One image software (Bio-Rad, UK). The net intensity of the band was normalized for the intensity of the band corresponding to 18 S RNA and the values were represented in the graph.

ELISA method for estimation of GSK-3*α*:

The hippocampi were homogenized in ice- cold 50 mM Tris-HCl buffer, pH 7.4 containing 150 mM NaCl, 2mM EDTA, 1 mM PMSF and 0.5% Triton X-100 followed by sonication (10 sec X 2 cycles). Homogenates were then centrifuged at 13,000 X g for 20 minutes. The clear extracts obtained were taken for quantitative measurement of GSK-3α by sandwich ELISA method as per manufacturer's instructions. The detection range of GSK-3α was 0.312-20 ng/ml with assay sensitivity of 0.108 ng/ml. The readings were measured at 450 nm in Tecan microplate reader. All the values obtained were expressed per mg total protein of tissue.

Immunocapture activity assay for IDE activity:

The activity of the captured insulysin was measured using a FRET substrate, Mca-GGFLRKHGQ-EDDnp where the scissile bond between R and K released the fluorophore from the quenching molecule and the fluorescence was measured at an excitation wavelength of 320 nm and an emission wavelength of 405 nm. All the values obtained were expressed per mg total protein of tissue lysate.

Stastical analysis:

The data are expressed as mean ± SD and differences between groups were analyzed by one-way ANOVA followed by post-hoc test using GraphPad Version 3 (Prizm; GraphPad Software Inc, San Diego, California, USA) and p values < 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Over the past few years, it is becoming increasingly evident that patients suffering from either type 1 diabetes [15] or type 2 diabetes [16] exhibit cognitive deficits and these changes may be associated with pathogenesis common to both diabetes and AD. Along these lines, since the last decade, insulin is being considered vital in the brain, where it is now recognized to be involved with several cerebral functions including synaptic plasticity, cognition and neuroprotection [17]. Insulin receptors are widely expressed throughout the human and rodent brain [18]. When stimulated by insulin, they trigger several signaling pathways, including the phospholipase Cγ, mitogen activated protein kinase, and phosphatidylinositol 3-kinase (PI3K) pathways. Our current study focused on the PI3K signal transduction pathway that regulates the activity of GSK-3. Dysregulation of GSK-3 activity has recently been linked to a number of neurodegenerative disorders [7]. The importance of the regulation of GSK-3 activity in neurons has been illustrated by studies showing that insulin-driven inactivation of GSK-3 by phosphorylation of the α and ß isoforms results in a relatively low phosphorylation state of the microtubule associated tau protein [19,20].

Influence of insulin on suppression of GSK-3*α* levels:

In the present study, STZ induced diabetic rats were used as a model system to study the modulation of the key insulin signaling pathway molecule viz., GSK-3α, which has strongly been correlated with facilitating the production of Aß. Towards this, adult female Sprague-Dawley rats were injected intraperitoneally with a single dose of streptozotocin (35 mg/ Kg). Measurement of glucose levels in the serum and CSF 3 days after STZ injection confirmed the hyperglycemic status in these animals [11]. After 4 weeks of onset of hyperglycemia, to the treatment groups, insulin (5 IU/day) was given intranasally for 6 consecutive days. Subsequently, quantitative analysis of the hippocampal extracts revealed a direct correlation between intranasal insulin administration and reduction in Aß levels [11]. These findings were in accordance with the previous reports on the influence of intranasal insulin in improving cognition and modulating peripheral Aß levels in early AD volunteers [13,21]. Further, we semi-quantitatively assessed the levels of GSK-3α by RT-PCR analysis (Fig. 1A). Neither diabetes nor insulin supplementation to the diabetic rats altered the transcription rate of GSK-3α. However, insulin treatment to the control rats indicated a small but

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ISSN: 0974 – 3987
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significant ($p < 0.05$) reduction in the mRNA level. Measurement of GSK-3α protein levels by ELISA (Fig. 1B) revealed that STZ induced insulin deficiency resulted in increased translation of GSK-3α which can be inhibited by administration of insulin (Fig. 1B). This increase in production especially in the absence of enhanced transcription rate of GSK-3α suggests the increase in half-life of GSK-3α mRNA in diabetic rats and insulin alters this stability, likely similar to its effect exerted in diabetic rat liver [22].

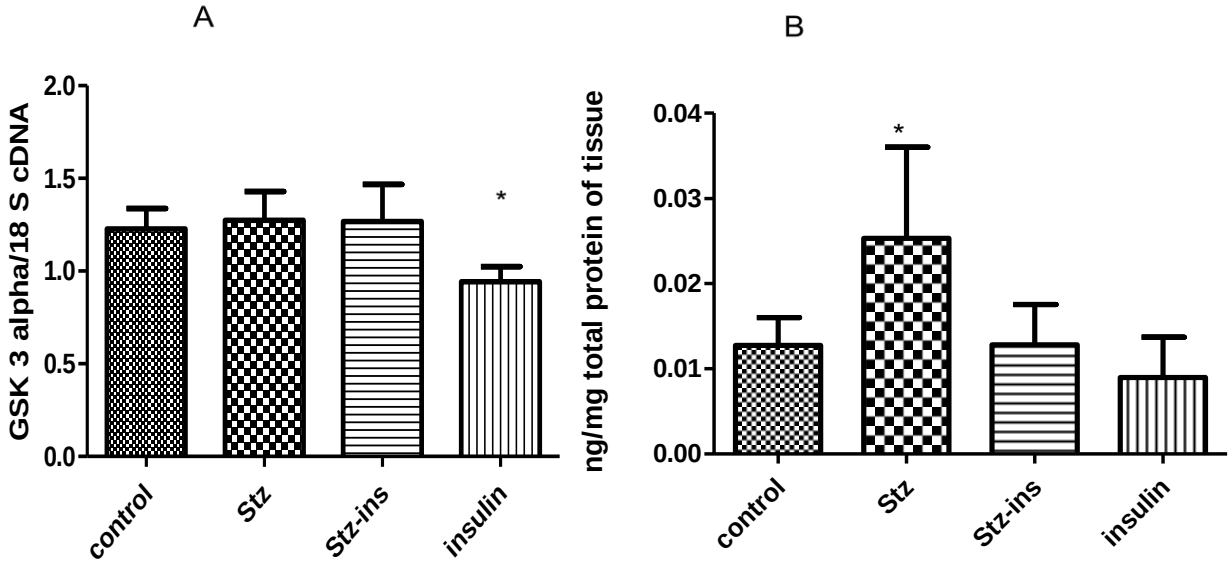

Fig. 1 Influence of insulin on expression of GSK-3α. (A) Levels of GSK-3α mRNA in the hippocampi dissected from control rats (Group 1), STZ treated rats (Group 2), STZ treated rats administered with intranasal insulin (Group 3) and control rats treated with insulin (Group 4), analyzed by RT-PCR and normalized to 18 S cDNA band intensity and represented graphically. (B) Amounts of GSK-3α measured in the hippocampal homogenates from control and treated animals using an ELISA assay for GSK-3α and represented per mg of total protein. Data are represented as mean ± SD from 5 animals for each group. *$p < 0.05$ considered as significant.

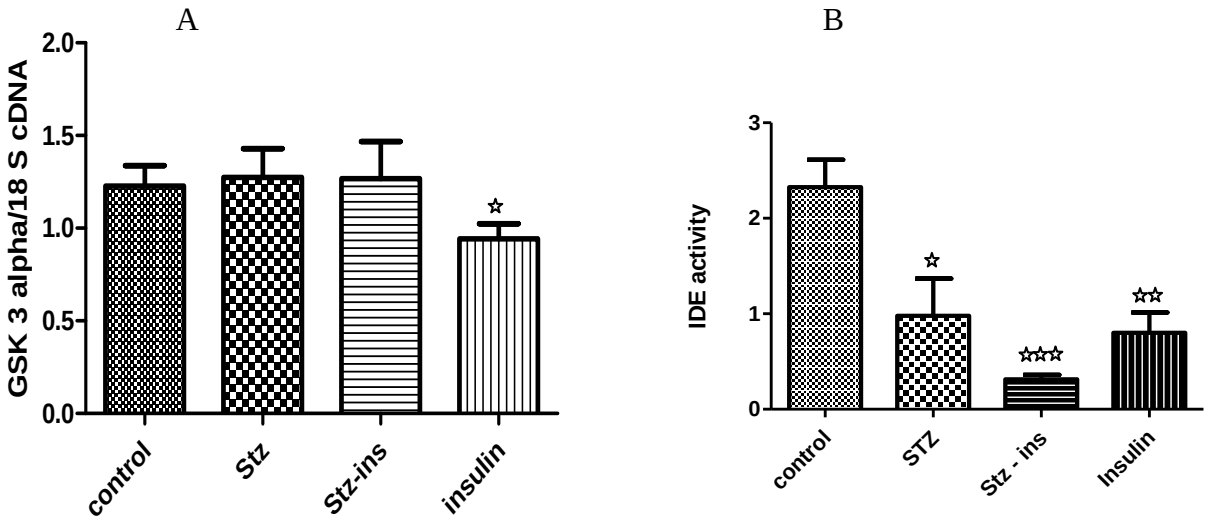

Fig. 2 Influence of insulin on expression of IDE. (A) Levels of IDE mRNA in the hippocampi dissected from control rats (Group 1), STZ treated rats (Group 2), STZ treated rats administered with intranasal insulin (Group 3) and control rats treated with insulin (Group 4), analyzed by RT-PCR and normalized to 18 S cDNA band intensity and represented graphically. (B) IDE activity measured in the hippocampal homogenates from control and treated animals using immunocapture fluorimetric assay and represented per mg of total protein. The sample size of n=5 was obtained from each group for analysis. The level of significance between the groups is *$p < 0.05$ considered as significant. **$p < 0.01$ and ***$p < 0.001$ considered more significant.

Effect of diabetes on the IDE activity and its mRNA expression and the response to insulin therapy:

Insulin degrading enzyme (IDE), a metalloprotease enzyme responsible for insulin degradation, has been demonstrated to play a key role in degrading Aß monomer both *in vivo* and *in vitro* [23,24] raising the possibility of upregulating IDE as an approach to reduce Aß. It is observed that IDE mRNA levels remained unaltered among control, STZ-treated diabetic rats and insulin supplemented diabetic rats. However, administration of insulin to control group resulted in marginal but significant ($p < 0.01$) decrease in IDE transcription (Fig. 2A). In contrast, IDE activity was considerably reduced ($p < 0.05$) in the STZ group with 5 weeks of insulin deficient diabetes (Fig. 2B). Treatment with insulin to either control group or STZ group showed further reduction in the protease activity of IDE (Fig. 2B). The inhibitory effect exerted by insulin on IDE is best explained by Song *et al* [25]. They have shown that IDE is oligomeric, undergoing a concentration dependent dimer-tetramer equilibrium and they proposed that the activating effect of peptide substrates is a result of their binding to one subunit and inducing a conformational change within the dimer. They have observed the activation of IDE with Aß and not with insulin. This was explained by suggesting that insulin, which is a dimer of A and B chains, can simultaneously bind to both subunits of the IDE dimer, with the A chain of insulin bound to one subunit and the B chain bound to the other subunit. This would preclude other peptide substrates from binding and activating the enzyme.

In summary, our data suggest that intranasally administered insulin acts via modulating GSK-3α and regulates the Aß levels in the hippocampus of streptozotocin induced diabetic rat model. This study adds an additional mechanism of action of insulin in the CNS which can pave the way in the development of AD therapeutics.

ACKNOWLEDGEMENTS

Financial assistance to SS from the Department of Science & Technology, New Delhi is gratefully acknowledged. MJ is a recipient of Junior Research Fellowship from Council of Scientific & Industrial Research, New Delhi, India.

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